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Pollinator shifts between *Ophrys sphegodes* populations: might adaptation to different pollinators drive population divergence?

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Running title: Local pollinator shift in *Ophrys sphegodes*

ABSTRACT

Local adaptation to different pollinators is considered one of the possible initial stages of ecological speciation as reproductive isolation is a by-product of the divergence in pollination systems. However, pollinator-mediated divergent selection will not necessarily result in complete reproductive isolation, since incipient speciation is often overcome by gene flow. We investigated the potential of pollinator shift in the sexually deceptive orchids *Ophrys sphegodes* and *O. exaltata* and compared levels of floral isolation versus genetic distance among populations with contrasting, predominant pollinators. We analysed floral hydrocarbons as a proxy for floral divergence between populations. Floral adoption of pollinators and their fidelity was tested using pollinator choice experiments. Inter-population gene flow and population differentiation levels were estimated using AFLP markers. The Tyrrhenian *O. sphegodes* population preferentially attracted the pollinator bee *Andrena bimaculata*, whereas the Adriatic *O. sphegodes* population exclusively attracted *A. nigroaenea*. Significant differences in scent component proportions were identified in *O. sphegodes* populations that attracted different preferred pollinators. High inter-population gene flow was detected, but populations were genetically structured at species level. The high inter-population gene flow levels independent of preferred pollinators suggest that local adaptation to different pollinators has not (yet) generated detectable genome-wide separation. Alternatively, despite extensive gene flow, few genes underlying floral isolation remain differentiated as a consequence of divergent selection. Different pollination ecotypes in *O. sphegodes* might represent a local selective response imposed by temporal variation in a geographic mosaic of pollinators as a consequence of the frequent disturbance regimes typical of *Ophrys* habitats.

Key words: adaptation, ecotypes, floral scent, gene flow, *Ophrys*, orchids, pollinator shift, sexual deception, speciation

INTRODUCTION

One important mechanism driving angiosperm diversification is pollinator-mediated selection due to pollen limitation and floral isolation (Lowry *et al.*, 2008; Xu *et al.*, 2011). Although the evolutionary role of pollinator-mediated selection remains debated under complex ecological conditions (Kay & Sargent, 2009), pollinator specialisation has traditionally been considered

one of the prime mechanisms for ecological speciation due to the direct relationship between pollinator specialisation and floral isolation (Grant, 1971; Johnson, 2006; Schiestl, 2012).

Pollinator shift frequency in specialised plant lineages depends on the influence of local selective forces (i.e. pollen limitation and pollen transfer efficiency), and the degree of floral isolation and its genetic basis (Xu *et al.,* 2012a; Schiestl & Schlüter, 2009, and references therein). In fact, if the transition to a different pollinator requires changes in several non-correlated floral traits and attraction signals, then strong and prolonged directional selection is required to promote those changes (Nosil *et al.,* 2009). Such evolutionary transition, involving several traits, will probably proceed gradually. Alternatively, some recently diverged lineages, where pollinator shifts facilitated rapid radiations, are often characterised by related species that attract different specific pollinators because of subtle changes in floral advertisement or shape, rather than large floral structural rearrangements and large changes in signals (Fulton & Hodges, 1999; Schemske & Bradshaw, 1999; Sturman *et al.,* 2004). These observations suggest that in such specialised plant groups only one or a few floral traits play a prominent role in preventing pollinator sharing, and that even small alterations in key traits may have substantial effects in attracting distinct pollinators, or pollinator groups.

Sexually deceptive orchids are typified by a combination of traits mediating specific pollinator attraction, and sexual mimicry of pollinator females achieves pollination specificity (Kullenberg, 1961; Paulus & Gack, 1990a; Bower, 1996). Several recent studies provide strong experimental evidence demonstrating that floral scent holds the key to specific pollinator attraction, while floral display serves a secondary role by increasing floral detection against vegetation background (Vereecken & Schiestl, 2009; Peakall *et al.,* 2010; Xu *et al.,* 2012a). Additional empirical evidence supports these results by showing that artificial manipulation of floral display does not significantly alter pollinator attraction (but see Spaethe *et al.,* 2007). In contrast, floral scent modifications increase or decrease pollinator visitation rates (Xu *et al.,* 2012b). Therefore, a subtle change in floral scent, even in the absence of marked changes in floral display, might potentially attract different orchid pollinators (Xu *et al.*, 2012a).

Recently, the genetic and chemical basis of pollinator specificity in sexually deceptive orchids has been partially elucidated. In *O. sphegodes* and related species, for instance, the specificity of pollinator attraction is due to quantitative variation in alkenes, which differ in double-bond position and carbon chain length (Mant *et al.,* 2005a; Schlüter *et al.*,2011a). Therefore, even minor variation in gene expression patterns underlying alkene biosynthesis

might be sufficient to produce a different odour bouquet, as in *O. sphegodes* versus *O. exaltata* (Schlüter *et al.*, 2011a; Xu *et al.* 2012b). Closely related species of sexually deceptive orchids in the same phylogenetic lineage attract different pollinators as a result of qualitative or only quantitative changes in proportions of active odour bouquet compounds. Alterations in compound proportions provide variability for selection to act upon, and thus offer the potential for ecological speciation (Schiestl & Ayasse, 2002; Xu *et al.*, 2012a).

Pollinator communities and species population sizes are likely to fluctuate through time, particularly in disturbed and transient habitats as those typically occupied by *Ophrys* species (Gardiner, 2009; Hutchings, 2010). Indeed, floral specialisation has been considered a risky strategy due to strict relationships between the survival/extinction of pollinator species, and the plant species dependent on its exclusive pollination service (Johnson & Steiner, 2000). Thus, potential interaction plasticity with the specialised pollinator might allow the plant to attain reproductive success, even under limited presence of the main pollinator, through temporary exploration of locally more abundant secondary pollinators (Waser & Ollerton, 2006). Under these conditions, local selection on plants imposed by a variable geographic and temporal mosaic of potential pollinators could lead to multiple pollination ecotypes (Harder & Johnson, 2009). This adaptive strategy might facilitate pollinator-specialised species survival when short-term pollinator community fluctuations occur as a consequence of habitat alterations, such as those caused by anthropogenic disturbance (Petanidou, 2008; Potts, 2010).

Whether *Ophrys* pollination ecotypes are populations, or incipient or actual ecological species (Van Valen, 1976) has been hotly debated among evolutionary biologists (*e.g.* Vereecken *et al.,* 2011; Bateman *et al.,* 2011). The crucial difference between adaptation to local pollinators (pollination ecotypes) and progenitor-derivative speciation due to a pollinator shift can be identified in the evolution of reproductive isolation associated with the transition to a different pollinator - a transition that is ultimately dependent on the strength of divergent selection and the amount of inter-population gene-flow (Schlüter *et al.,* 2011b).

Ultimately, biological species are defined by reproductive isolation, which is typically weak or absent among geographical races (Slatkin, 1987; Coyne & Orr, 1998). Therefore, reduction or loss of gene flow (as consequence of the insurgence of some forms of reproductive isolation) between ancestral populations leads to incipient ecological species from local ecotypes, allowing their gene pools to develop independently (Macnair & Gardner, 1998). Nevertheless, since local adaptation often represents the initial stages of ecological speciation, a marked distinction between these sequential stages, based on the level of gene

flow between different ecotypes, is often difficult to define (Nosil, 2012). Lexer & Widmer (2008) emphasised the seriousness of this challenge in several ecological species pairs that displayed a considerable amount of inter-specific gene flow, and where genomic divergence was only detected at a few loci despite using several genome-wide markers.

To date, few studies have examined the nature and level of reproductive isolation between different ecotypes of a given species (Scopece *et al.*, 2010, and references therein) and geographic isolation is considered the primary isolating factor among races or local ecotypes (Lowry *et al.* 2008). However, it does not represent an obvious barrier that prevents gene flow between geographically close or adjacent ecotypes/populations (Anderson *et al.,* 2010), a situation that can in principle allow constant gene flow between populations (Räsänen & Hendry, 2008). In such cases, the amount of floral isolation is comparable with the levels of inter-population gene flow as an indirect estimate of whether different populations represent locally adapted pollination ecotypes that established a form of incipient reproductive isolation (Nosil *et al.,* 2009; Thibert-Plante & Hendry, 2010). In fact, although divergent selection can favour reproductive isolation at a local scale, incipient divergence can be prevented by the homogenising effect of gene flow between adjacent populations, maintaining species cohesion (Morjan & Rieseberg, 2004). Investigations into transient stages in the *continuum* between local adaptation and incipient speciation are integral to speciation research. Such studies may serve to identify the selective forces acting on floral traits, and establish the evolutionary outcomes of pollinator shift and subsequent floral isolation (Fenster *et al.,* 2004).

In the present study, with the aim of disclosing the effect of changes in preferred pollinators on the establishment of reproductive isolation, we investigated the potential for pollinator shifts in the Mediterranean sexually deceptive orchid *Ophrys sphegodes* MILL., and compared levels of floral isolation *versus* genetic distance between populations with different, dominant pollinators. We estimated intra- and inter-specific reproductive isolation and gene flow in *O. sphegodes s.s.* and the closely related species *O. exaltata* TEN. The two taxa belong to the same phylogenetic lineage, and Xu *et al.* (2011) only recently inferred that the species are effectively reproductively isolated.

MATERIALS AND METHODS

STUDY SYSTEM

The sexually deceptive orchid species *Ophrys sphegodes* and *O. exaltata* were chosen for this study. *Ophrys sphegodes* is a widespread species that inhabits sunny meadows or calcareous

grasslands from the British Isles to the southern Mediterranean countries (Delforge, 2005) whereas *O. exaltata* is more restricted to the Mediterranean region, inhabiting sandy soils in southern France and the central part of the Mediterranean region. In a broad survey of the entire genus, Devey *et al.* (2008) showed that the two species are phylogenetically closely related. In peninsular Italy, *O. sphegodes* and *O. exaltata* frequently occupy similar ecological niches - primarily coastal sandy areas (Delforge, 2005) - and the taxa often occur in 'mosaic sympatry' (*sensu* Mallet *et al.,* 2009). Due to severe habitat fragmentation, particularly where the Apennine mountain chain separates the Tyrrhenian and Adriatic coasts, both species accommodate a large number of local varieties, either interpreted as local ecotypes or endemic species (*cf.* Delforge, 2005; Pedersen & Faurholdt, 2007). Overall, the two species exhibit well-established reproductive isolation via different primary pollinators (i.*e. Andrena nigroaenea* for *O. sphegodes* and *Colletes cunicularis* for *O. exaltata*). However, the species are interfertile and can produce hybrids in the wild (Xu *et al.,* 2011).

GENETIC ANALYSES

We investigated four *O. sphegodes* populations and three *O. exaltata* populations along the Italian peninsula (Table 1). For each plant sampled, a piece of leaf tissue was field collected and placed in a plastic bag filled with silica gel. Genomic DNA was extracted using GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, Italy). The amplified fragment length polymorphism (AFLP) procedure followed Vos *et al.* (1995), albeit with modifications (Moccia *et al.* 2007) and using fluorescent dye-labelled primers. Six primer combinations were chosen from Xu *et al.* (2011): FAM-*Eco*RI-AGC/*Mse*I-ACAC, NED-*Eco*RI-ACC-/*Mse*I-ACTG, HEX-*EcoRI*-AGC/*Mse*I-ATCG, FAM-*Eco*RI-ATG/*Mse*I-CGG, NED-*Eco*RI-AAC/*Mse*I-CGC, and HEX-*Eco*RI-AGC/*Mse*I-CCAA. Fragment separation and detection were conducted on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA). GeneScan-500 LIZ (Applied Biosystems, Foster City, USA) was used as the internal standard. Raw data alignment and fragment-size detection were performed using GeneMapper 3.7 software (Applied Biosystems, Foster City, USA). Presence or absence of AFLP bands was scored visually. Artefacts and missing data were avoided by only including informative AFLP markers in the binary matrix that could be unambiguously scored for all samples.

GenAlEx (Peakall & Smouse, 2006) was run as the macro in Microsoft Excel to calculate genetic distances as the basis for generating a principle coordinate analysis (PCoA) scatter plot. An analysis of molecular variance (AMOVA) was conducted using a matrix of genetic distances between all haplotype pairs. Genetic differentiation was estimated using Φ-

statistics, an *F*-statistics analogue for binary data. Significance of Φ-statistics and variance components were assessed with 999 permutations (Peakall & Smouse, 2006). Pairwise Φ_{ST} values based on Jaccard's similarity were calculated from FAMD (Schlüter & Harris, 2006).

STRUCTURE 2.2 (Pritchard *et al.,* 2000) was applied to investigate population structure. The model implemented in STRUCTURE set the posterior probability (q) to describe the proportion of an individual genotype originating from each of K categories. Following the method described in Evanno *et al.* (2005), we tested K from 1 to 7 (*i.e*. the number of populations sampled) with a burn-in of 50 000 steps, followed by 300 000 MCMC iterations and 10 replicates to confirm stabilisation of summary statistics. Estimates were carried out under the admixture model, allowing for correlated allele frequencies on all sampled individuals, while ignoring sampling localities. The output obtained from STRUCTURE was graphically displayed with DISTRUCT (Rosenberg, 2004). The genomic basis of differentiation between *O. sphegodes* populations from Cuma and Gargano was analysed using a genome scan. Accordingly, to identify putative genomic targets of divergent selection, an F_{ST} outlier scan was conducted using the Dfdist package (Beaumont & Nichols, 1996), which applies the Bayesian allele frequency estimate method developed by Zhivotovsky (1999). The analysis was conducted as in other studies (*e.g.* Minder & Widmer, 2008; Pérez-Figueroa *et al.,* 2010), excluding loci with an allele frequency over 0.99, and using mean F_{ST} trimmed at the 30% level. This trimmed mean F_{ST} was chosen as the target average for Dfdist to simulate F_{ST} null distribution values (50000 realisations), assuming a θ parameter of 0.05; variation in this θ parameter was reported to have very little impact on results (*e.g.* Minder & Widmer, 2008, and references therein). F_{ST} outliers were identified as data points outside the 95% confidence interval (outside the 0.025 and 0.975 quartiles, and at $P < 0.025$).

The potential effects of geography and pollinator differentiation on genetic structure within *O. sphegodes* were separated using a generalised linear model (GLM). Pairwise Φ_{ST} values were modelled with the explanatory variables geographic distance and shared pollinator, as well as an interaction term between the two variables. A geographic distance matrix was computed with ArcGIS 9.2. The analysis was performed in R 2.15.2 (R Development Core Team, 2012), as described in Schlüter *et al.* (2011b).

POLLINATOR CHOICE EXPERIMENTS

Pollinator fidelity was tested through pollinator choice experiments between *O. sphegodes* and *O. exaltata* individuals. Plant inflorescences were collected from natural populations

along the Tyrrhenian (Cuma, Campania) and Adriatic (Gargano, Puglia) coasts where *O. sphegodes* and *O. exaltata* are sympatric. The study populations co-flower at the end of March/beginning of April. Pollinator choice plots with individuals of both species from the two coasts were established at Cuma and Gargano. We established 12 choice-plots from 17th to 25th of March 2011 available during a two hour period from 09.00 to 11.00 h; five choice-plots were established in Cuma, seven plots in Gargano, intermixed with natural populations. Each plot consisted of four inflorescences, with one individual at a time of each species from both coasts. The inflorescences were randomly placed, 30 cm apart, in flowering bushes along sandy paths. Inflorescences in the choice plots were replaced with new ones after every pollination event or, in the absence of any visit, after 30 min.

Male bees patrolled along sandy footpaths, where numerous nesting places of female solitary bees were observed; the male bees also checked for females in flowering shrubs (*e.g. Rosmarinus officinalis*, *Spartium junceum*, *Emerus major*) where the female bees foraged for nectar. Pollination events were recorded only when the bee was successfully caught after an observed pseudo-copulation leading to pollinarium removal. The bees were later identified by comparison to an *Ophrys* pollinator reference collection at the University of Zürich, Switzerland.

An additional pollinator-baiting experiment was performed from 10.00 to 13.00 h on the 21st of March 2012 on the Tyrrhenian coast by exposing freshly cut inflorescences of O.sph$_{CUMA}$ during local pollinator hours. Visiting bees were caught only after observation of pseudo-copulation with pollinia removal, and inflorescences were replaced following each pollination event.

SCENT ANALYSES

Floral hydrocarbons produced by the two species were analysed for plants from all populations for which pollinator choice experiments were performed, *i.e. O. sphegodes* from Cuma, Tyrrhenian Coast (O.sph$_{CUMA}$) and Gargano, Adriatic Coast (O.sph$_{GAR}$), and *O. exaltata* from Cuma, Tyrrhenian Coast (O.exa$_{CUMA}$) and Gargano, Adriatic Coast (O.exa$_{GAR}$). For each sampled individual plant (the same plant used for genetic analyses, plus additional individuals bearing up to 20 flowers), one labellum of an unpollinated flower was placed in a 2 ml glass vial (Supelco) and rinsed in 500 µl hexane (HPLC grade, Fluka) for one minute while gently shaken. The labellum was subsequently removed from the vial; all scent samples were stored at -20° C until analysis. Gas chromatography (GC) was performed following Mant *et al.* (2005b) with minor modifications as detailed in Xu *et al.* (2011). Several samples

were re-analysed for compound identification with a mass selective detector (GC/MSD; Agilent 5975) using the same oven and column parameters. It is noted that (*Z*)-11 and (*Z*)-12 alkenes cannot be discriminated with the parameters used. Compound spectrum and retention time were compared with those of a synthetic standard, as reported by Xu *et al.* (2011). The relative amount of each odour compound was calculated as the proportion of total alkene and alkane amounts with a chain length between 18 and 30 carbons. Principal component analysis (PCA) was used for analysis of inter-species floral scent variation based on scaled relative amount of hydrocarbons. The within species differences in floral scent bouquet between populations were analysed using distance-based tests for homogeneity of multivariate dispersions (Anderson, 2006), with 999 permutations. The differences for each individual compound and total alkenes between different populations within species were analysed using student t-test or Mann–Whitney U test, depending upon the results of testing for normality and heteroscedasticity of variance using Leven's and Shapiro-Wilk tests. All statistical analyses for floral scent were performed in R 2.15.2 (R Development Core Team, 2012).

RESULTS

GENETIC ANALYSES

AFLP analysis yielded 322 variable markers. Genetic divergence between population pairs was assessed via Φ_{ST} values, yielding the following results: the lowest Φ_{ST} values were detected between $O.sph_{ARG}$ and $O.sph_{CLA}$ (0.07) and the highest between $O.sph_{ARG}$ and $O.exa_{TUS}$ (0.27); $O.sph_{CUMA}$ and $O.sph_{GAR}$ populations showed a Φ_{ST} of 0.10. Average Φ_{ST} was 0.14 between *O. sphegodes* populations, and 0.18 between *O. exaltata* populations. Interspecific Φ_{ST} between *O. sphegodes* and *O. exaltata* populations averaged 0.21 (Table 2, Fig.1). AMOVA showed that 87% of genetic variance was partitioned among individuals within a population, while the remaining 13% was explained by variance among populations (Table 3).

PCoA analysis grouped allopatric and sympatric populations based on taxonomic classification (the first two axes explained 25.9% and 19.4% of the variation, respectively; Fig. 2). Only a few individuals from sympatric Cuma and Gargano populations were intermixed between the two groups. PCoA showed that all *O. sphegodes* populations from the Tyrrhenian Coast ($O.sph_{CLA}$, $O.sph_{ARG}$ and $O.sph_{CUMA}$) formed a cohesive group, but the species populations also largely overlapped with the $O.sph_{GAR}$ Adriatic population.

The most probable number of genetic clusters (*K*) present in the data, (determined following Evanno *et al.* 2005) was *K*=2, corresponding to the assumption that only two

species contributed to the sample gene pool. Individuals of the two species exhibited strong assignment to their respective cluster, with the exclusion of a few putative hybrid individuals (Fig. 3).

A genome scan for F_{ST} outliers was performed for *O. sphegodes* populations from Cuma and Gargano by using a trimmed mean F_{ST} of 0.027 (Fig. 4). Overall, 269 loci were included in the genome scan, which identified four outliers ($P < 0.025$) below or above the respective 0.025 or 0.975 quartiles of the expected F_{ST} distribution. All four outlier loci were more strongly differentiated than expected. However, among the four loci, only two were unique to the comparison between *O. sphegodes* populations from Cuma and Gargano, while the other two loci were found as outliers also in other pairwise population comparisons.

Our GLM analysis modelled genetic pair-wise population differentiation (Φ_{ST}) as a function of geography and shared pollinators. Geography and pollinators were both significant ($P < 0.05$) factors in explaining population differentiation (Table 4).

POLLINATOR CHOICE EXPERIMENTS

Pollinator activity was negligible in the afternoon, and overall activity in Gargano was notably higher than that in Cuma. The captured pollinator summary is given in Table 5. O.exa$_{GAR}$ attracted 10 *C. cunicularius* individuals and O.sph$_{GAR}$ attracted four *A. nigroaenea* individuals; both taxa from Gargano attracted their legitimate pollinators independent of where the plots were located (Gargano and Cuma). Results differed for the two Cuma species. O.exa$_{CUMA}$ attracted four *C. cunicularius* individuals, and two individuals of a yet unidentified bee species in the genus *Eucera*. The *Eucera* bees were only captured in the *O. exaltata* Cuma population, and it is the first time a *Eucera* bee has been reported to pollinate individuals from the *O. sphegodes/exaltata* lineage. O.sph$_{CUMA}$ attracted five *A. nigroaenea* individuals and 46 *A. bimaculata* individuals. The pollinator-baiting experiment performed in 2012 confirmed that O.sph$_{CUMA}$ attracts *A. bimaculata* and *A. nigroaenea*. These species were caught six times and twice, respectively, on freshly cut inflorescences.

SCENT ANALYSES

The PCA plot of *O. exaltata* and *O. sphegodes* from the Tyrrhenian and Adriatic coasts showed a clear separation between the two species (Fig. 5), consistent with previous studies (Xu *et al.* 2011). The distance-based tests for homogeneity of multivariate dispersions showed that *O. sphegodes* populations from the Tyrrhenian and Adriatic coast were significantly different in their floral scent bouquet (p=0.023; 999 permutations). In contrast,

the floral scent bouquet of *O. exaltata* was not significantly different between two populations (p=0.209; 999 permutations). These floral scent differences were mainly due to a higher proportion of total alkenes in Tyrrhenian populations (Fig. 6). For *O. sphegodes*, the $O.sph_{CUMA}$ population produced 62.8 ± 5.2 % total alkenes, whereas the $O.sph_{GAR}$ population only produced 38.7 ± 15.0 % total alkenes ($p < 2.2 \cdot 10^{-16}$, Mann–Whitney U test). For *O. exaltata*, the $O.exa_{CUMA}$ and $O.exa_{GAR}$ populations produced 69.3 ± 8.0 % and 59.3 ± 16.2 % alkenes respectively (p=0.019, Mann–Whitney U test) (Fig. 6). It is noticeable that the $O.sph_{CUMA}$ population not only produced a higher proportion overall of alkenes that are active compounds to *A. nigroaenea* (the pollinator of *O. sphegodes*) but also produced three compounds, (*Z*)-7-C_{21}, (*Z*)-7-C_{23} and (*Z*)-7-C_{25}, that are active compounds to the pollinator of *O. exaltata*, *C. cunicularius* (Mant *et al.*, 2005a), but were absent from the $O.sph_{GAR}$ population.

DISCUSSION

Overall, our results indicated a pollinator shift, and showed significant differences in floral scent between populations of *O. sphegodes*. However, significant intra-specific genetic structuring was not observed between populations. These results suggest the following: (a) the Cuma and Gargano *O. sphegodes* populations have reached an early stage of divergence, and are adapting to different pollinator species; or (b) plant-pollinator relationships in some sexually deceptive orchid species are more geographically variable than has traditionally been proposed (*i.e.* Kullenberg, 1961).

Our previous pollinator choice experiments with *O. sphegodes* and the sympatric *O. exaltata* in semi-natural/disturbed habitats along the Tyrrhenian and Adriatic Italian coasts indicated the absence of pollinator sharing between these two closely related species (Xu *et al.,* 2011). However, our genetic analyses, even if with a small data set, supported previous findings showing that hybridisation can sporadically occur (Xu *et al.*, 2011). Interestingly, our experiments also revealed that the Tyrrhenian coast *O. sphegodes* ($O.sph_{CUMA}$) was pollinated primarily by the bee species *A. bimaculata. Andrena nigroaenea* is the typical *O. sphegodes* pollinator throughout most of the species range (Mant *et al.,* 2005b), but this species was only responsible for approximately 10% of pollinia removal at Cuma (Table 5). More importantly, *O. sphegodes* from the Tyrrhenian coast ($O.sph_{CUMA}$) maintained its attractiveness to *A. bimaculata* when transferred to Gargano (Adriatic Coast), indicating that *A. bimaculata* attraction is based on specific floral traits - probably floral scent - and not merely on the

(potentially) more frequent occurrence of this bee species at Cuma. To date, *A. bimaculata* has only been reported as a pollinator of the typically abdomen-pollinated *O. sicula* (Gaskett, 2010), and as a possible pollinator of *O. creticola* on Crete (Paulus & Gack, 1990b; Paulus & Schlüter, 2007).

Floral scent analysis results indicated stronger differentiation between the *O. sphegodes* populations than between the *O. exaltata* populations from the two coastal regions (Figs. 5, 6) and such differences were mainly due to a higher proportion of alkenes produced by the $O.sph_{CUMA}$ population. Three compounds [*i.e.* (*Z*)-7-C_{21}, (*Z*)-7-C_{23} and (*Z*)-7-C_{25}] that are active to *C. cunicularius* (preferred pollinator of *O. exaltata*) were exclusive to the $O.sph_{CUMA}$ population (Fig. 6). These three (Z)-7 alkenes have not previously been recorded as an EAD-active compound for *A. nigroaenea* (Stökl *et al.,* 2005), and the addition of a (*Z*)-7 alkene mix [made up of (*Z*)-7-C_{21}, (*Z*)-7-C_{23} and (*Z*)-7-C_{25}] rendered *O. sphegodes* flowers less attractive to *A.* possible role played by these three compounds in *A. bimaculata* attraction has yet to be tested. Interestingly, the Tyrrhenian *O. sphegodes* ($O.sph_{CUMA}$) scent continued to attract *A. nigroaenea*, even though its scent bouquet was different both qualitatively and quantitatively from that of the Adriatic *O. sphegodes* ($O.sph_{GAR}$). Therefore, the two *O. sphegodes* populations share *A. nigroaenea* as pollinator, and no substantial floral isolation should be expected between them.

In spite of the small genetic differences, our data showed that between the seven study populations, levels of intra-specific gene flow were higher than inter-specific levels. Overall, lower genetic divergence was detected between allopatric *O. sphegodes* populations than between sympatric *O. sphegodes* and *O. exaltata* populations. Despite the different pollinators attracted by each species, the Adriatic *O. sphegodes* ($O.sph_{GAR}$) population exhibited a lower genetic distance from the geographically closer $O.sph_{CUMA}$ population than from the more distant $O.sph_{CLA}$ and $O.sph_{ARG}$ Tyrrhenian populations. $O.sph_{CLA}$ and $O.sph_{ARG}$ probably attract the same pollinator as the Adriatic *O. sphegodes*, *i.e. A. nigroaenea*. Nevertheless, GLM results suggested that geography and shared pollinators were significant factors in differentiation between *O. sphegodes* populations. Despite the difficulties in resolving ancestral polymorphisms due to hybridisation in very recently diverged populations/species, the low genetic differentiation estimates (*i.e.* low Φ_{ST} values; Table 2) observed between different *O. sphegodes* populations were comparable with, or even lower than, the values previously reported between other populations of *Ophrys* species using, as in our study, AFLP markers (Devey *et al.,* 2009; Schlüter *et al.,* 2011b). Therefore, data suggest that *O. sphegodes* populations continue exchanging alleles despite an apparent change in preferred

pollinator species. Nevertheless, the genome scan for F_{ST} outliers identified four loci that were more strongly differentiated than expected by chance. Among these four loci, two were consistent with a scenario of divergent selection between *O. sphegodes* populations from Cuma and Gargano, even if the small sample set and the absence of multiple pairwise comparisons between populations with the same pollinator combination strongly limit the power of this analysis.

Based on high inter-population gene flow in *O. sphegodes*, independent of pollinators, we can infer that local adaptation by pollinator shift in the Tyrrhenian *O. sphegodes* populations has not (yet) generated detectable genome-wide separation from the Adriatic populations. In an alternative (but not mutually exclusive) scenario, the Tyrrhenian and Adriatic populations represent a step towards incipient ecological speciation driven by genic divergence (*i.e.* loci linked to F_{ST} outliers). In this case, even if the entire genome is experiencing gene flow, as long as the one or few genes underlying the floral isolation trait remain differentiated as a consequence of divergent selection, these populations have the potential to maintain reproductive isolation, regardless of gene flow between them (*i.e.* genic ecological speciation model and porous genomes; Wu, 2001; Lexer & Widmer, 2008).

Our results match studies by Paulus & Gack (1990b), Lorella *et al.,* (2002) and Claessens & Kleynen (2011) that demonstrate a (narrow) range of related *Ophrys* species (particularly those with a wide distribution) pollinated by different bees, and suggest that *Ophrys* - pollinator interactions are flexible rather than a static one-to-one relationship. Vereecken *et al.* (2011) observed this flexibility in *Ophrys* - pollinator relationships, and Bower (1996) reported that so-called "minor responders" often accompany the main pollinator(s) in the sexually deceptive Australian orchid genus *Chiloglottis*. Our finding that a *Eucera* bee species serves as a minor *O. exaltata* pollinator in the Cuma population suggests that even bees of other genera can act as secondary pollinators.

The different pollination ecotype resolved in the Tyrrhenian *O. sphegodes* population may represent a response to local selection imposed by variability in the geographic mosaic of pollinators. Genetic drift is an alternative explanation for the observed pattern in floral odour bouquet evolution, and its attractiveness to a novel pollinator in the Tyrrhenian populations. However, the low between-population genetic differentiation (Table 3) is indicative of extensive gene flow. In addition, the large effective population size typical found in these terrestrial deceptive orchids (Cozzolino & Widmer, 2005 and reference therein), including our study system (Mant *et al.,* 2005b), indicates that a drift scenario is less likely compared with an adaptive process of pollinator shift.

A classical question in the evolution of specialised pollination mechanisms is how much stringency in the relationship between a plant species and its pollinators can limit the ability of a plant species to adapt to and survive local and temporal changes in the pollinator community. Interestingly, a few studies on the spatial and temporal breadth of pollinator of *Ophrys* taxa (including this study) have revealed other, minor pollinators. An imperfect match in odour between the plant mimic and its model (the female insect) may represent a form of pre-adaptation for a local or temporary shift in specialised pollinators (Bower, 1996; Peakall *et al.,* 2010). Indeed, bioassays have shown that sexual deception is a type of imperfect mimicry, the pollinator bees actively preferring "novel" signals over the more commonly encountered cues as adaptive responses to promote outbreeding (Vereecken *et al.,* 2008). Therefore, compound variability involving compounds inactive to some pollinators but active to others, and negative frequency-dependent selection, might provide an advantage to the uncommon odour phenotypes by increasing pollination success. Such selection for imperfect mimicry may facilitate more relaxed signal refinement in mimics to optimally match the signals released by the mimic's specific insect models. It would thereby maintain a source of natural inter-individual variation in floral scent, which could represent the standing genetic variation necessary for rapid local adaptive responses to a fluctuating pollinator community in disturbed habitats.

Although compelling evidence supports floral adaptation as the basis for ecological speciation (Kay & Sargent, 2009), a local shift to a different pollinator does not necessarily lead to speciation, particularly if the selective pressure is transient or fluctuating. *Ophrys* populations adapted to different pollinators may well represent a very interesting case-study of incipient speciation by local adaptation. How easily this transient change in preferred pollinators between locally adapted populations can turn into a "permanent" form of reproductive isolation and speciation probably depends on the tempo and mode of divergent selective forces working on local populations, and on the degree and duration of the disturbances generating the local ecological differences.

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640 TABLE 1. Examined populations of *O. exaltata* and *O. sphegodes*.

641

Species	Sample Size	Origin	Date	Collector
O. exaltata ($O.exa_{GAR}$)	17	Capoiale, Gargano, Puglia, Italy	Mar-09	H. Breitkopf
O. exaltata ($O.exa_{TUS}$)	10	Marina di Castagneto, Tuscany, Italy	Apr-09	H. Breitkopf
O. exaltata ($O.exa_{CUMA}$)	15	Cuma, Campania, Italy	Mar-09	H. Breitkopf
O. sphegodes ssp. *classica* ($O.sph_{CLA}$)	12	Porto San Stefano, Tuscany, Italy	Mar-10	R. Souche
O. sphegodes ($O.sph_{CUMA}$)	15	Cuma, Campania, Italy	Mar-09	H. Breitkopf
O. sphegodes ($O.sph_{GAR}$)	17	Capoiale, Gargano, Puglia, Italy	Mar-09	H. Breitkopf
O. sphegodes ssp. *argentaria* ($O.sph_{ARG}$)	9	Caldine-Fiesole, Tuscany, Italy	Apr-09	H. Breitkopf

642

643

TABLE 2. Pairwise Φ_{ST} values (Coefficient: Standard Jaccard. Distance Transformation: d=1-s) for the examined populations.

$O.sph_{ARG}$	$O.sph_{CLA}$	$O.exa_{TUS}$	$O.sph_{GAR}$	$O.exa_{GAR}$	$O.sph_{CUMA}$	$O.exa_{CUMA}$
0.00						
0.07	0.00					
0.28	0.24	0.00				
0.17	0.16	0.23	0.00			
0.27	0.25	0.20	0.13	0.00		
0.18	0.17	0.24	0.10	0.19	0.00	
0.26	0.25	0.16	0.21	0.16	0.17	0.00

Table 3 Analysis of molecular variance (AMOVA) for AFLP markers (d.f.: degree of freedom; SSD: sum of squared deviations; %: proportion of variance components, standard error ≤2.93%; Φ: genotypic variation; * $P \leq 0.001$).

Source of variation	d.f.	SSD	%	Φ
Among all populations	6	603.9	13	0.133*
Within all populations	87	2861.7	87	
Among *O.sphegodes* populations	3	231.2	10	0.098*
Within *O. sphegodes* populations	48	1548.7	90	
Among *O.exaltata* populations	2	193.9	12	0.121*
Within *O.exaltata* populations	39	1313.0	88	

668 TABLE 4. Generalised linear model of pairwise Φ_{ST} values among *O. sphegodes* populations
669 as explained by the factors indicated. To facilitate the analysis, the Cuma population was
670 assumed to have a different pollinator than the remaining *O. sphegodes* populations.

Factor	*Estimate*	*S.E.*	*t-value*	*p-value*	*Significance*
Intercept	0.12636	0.00642	19.672	0.00257	**
Geography	0.00639	0.00088	7.232	0.01859	*
Shared Pollinator	-0.53657	0.10196	-5.263	0.03426	*
Geography × Shared Pollinators	0.09420	0.01817	5.184	0.03525	*

671 Significance: *, $p<0.05$; **, $p<0.01$.

672

673

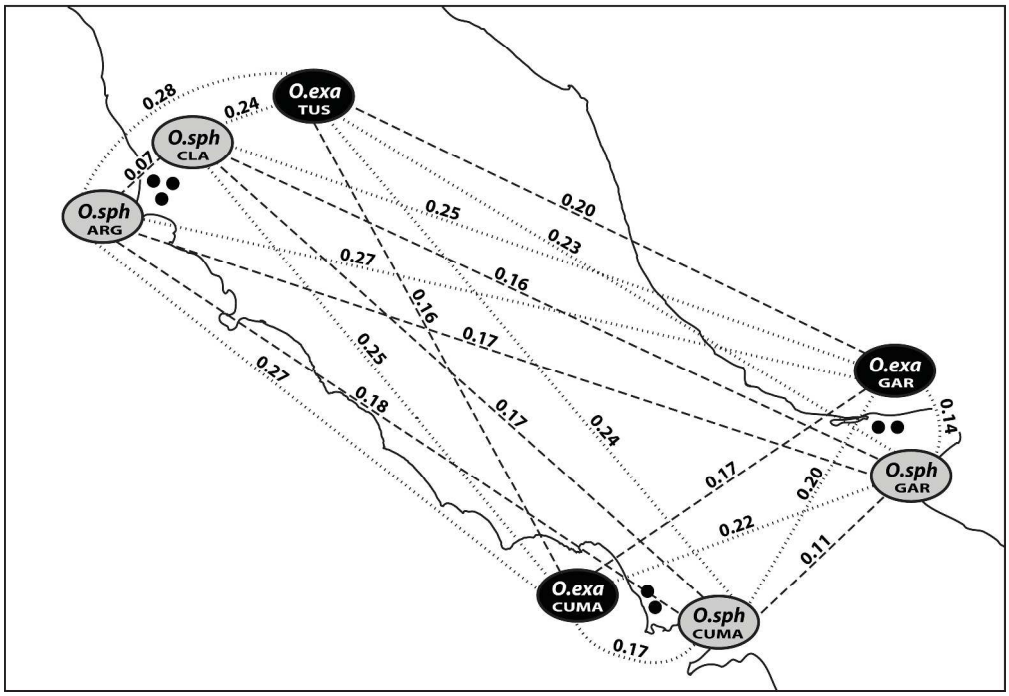
674 TABLE 5. Pollinator choice experiment: Bee counts. CUMA, Campania, Tyrrhenian Coast;

675 GARGANO, Puglia, Adriatic Coast.

		O. sphegodes CUMA	*O. sphegodes* GARGANO	*O. exaltata* CUMA	*O. exaltata* GARGANO
Cuma choice-plots	*A. bimaculata*	4			
	A. nigroaenea	1	2		
	C. cunicularius				2
	Eucera sp.			2	
Gargano choice-	*A. bimaculata*	42			
plots	*A. nigroaenea*	4	2		
	C. cunicularius			4	8
	Eucera sp.				
all choice-plots	*A. bimaculata*	46 (4+42)			
(Cuma+Gargano)	*A. nigroaenea*	5 (1+4)	4 (2+2)		
	C. cunicularius			4 (0+4)	10 (2+8)
	Eucera sp.			2 (2+0)	

676
677

678 FIGURE LEGENDS
679
680 FIG. 1. Map of Central Italy with Φ_{ST} values plotted for pairwise population comparisons.
681 Black dots indicate the locations of sympatric populations of *O. sphegodes* and *O. exaltata* in
682 the Italian regions of Tuscany, Campania and Apulia.
683
684 FIG. 2. PCoA plot based on individual genetic distances calculated from 322 polymorphic
685 AFLP markers. The first two axes explained 25.9% and 19.4% of the variation, respectively.
686
687 FIG. 3. Summary of population structure in *O. exaltata* and *O. sphegodes* using Bayesian
688 assignment analysis for a K= 2 model. Most individuals from *O. sphegodes* populations
689 showed strong assignment probabilities associated with cluster 1 (dark grey), whereas
690 specimens from *O. exaltata* were classified with cluster 2 (light grey). Two putative hybrid
691 individuals showing admixed proportions were allied with the *O. sphegodes* population from
692 Cuma.
693
694 FIG. 4. Dfdist plot of F_{ST} values against heterozygosity estimates for the *O. sphegodes*
695 population pair ($O.sph_{CUMA}$ and $O.sph_{GAR}$). Each circle indicates an AFLP marker. The
696 lower, intermediate and higher lines represent 5%, 50% and 95% confidence intervals,
697 respectively. Outlier loci ($P < 0.025$) are labelled and displayed as black dots.
698
699 FIG. 5. PCA plot of floral scent derived from sympatric *O. sphegodes* and *O. exaltata*
700 populations.
701
702 FIG. 6. Scent profiles, from sympatric *O. exaltata* ($O.exa_{CUMA}$ and $O.exa_{GAR}$), and *O.*
703 *sphegodes* ($O.sph_{CUMA}$ and $O.sph_{GAR}$) populations.
704



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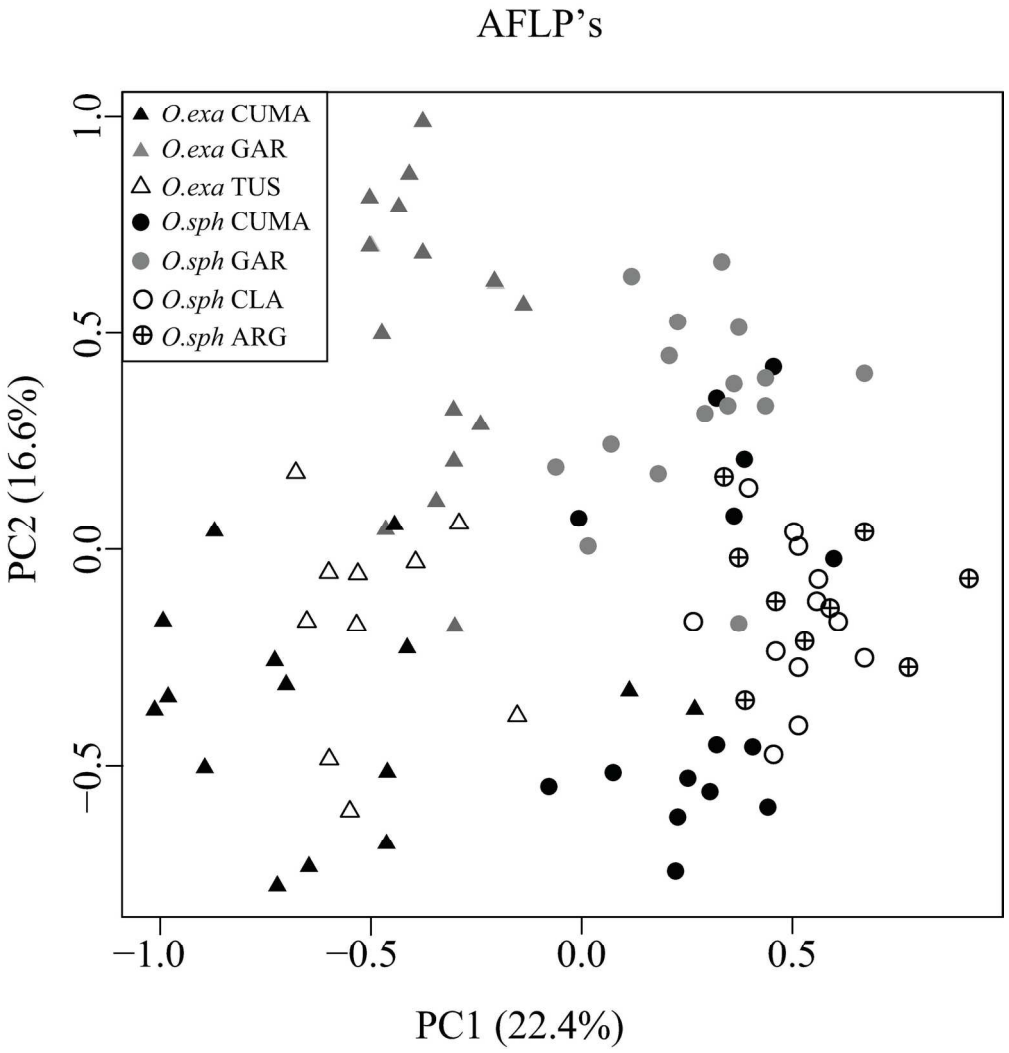
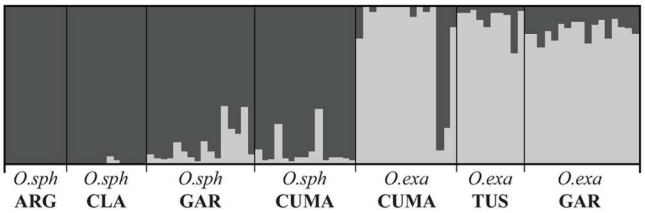
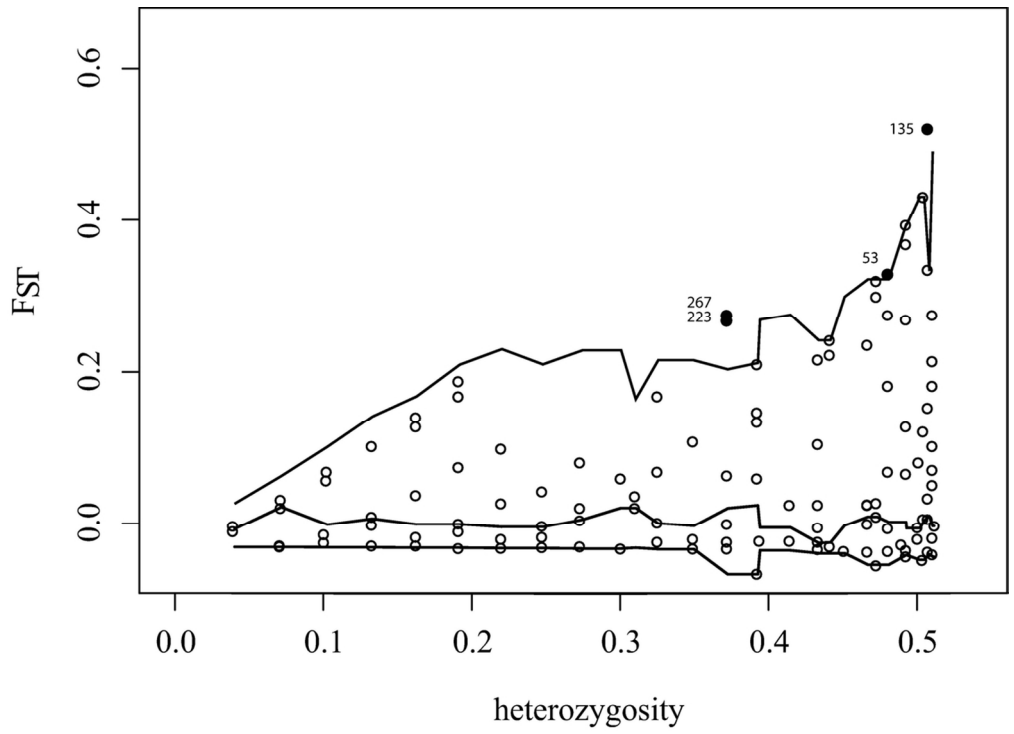


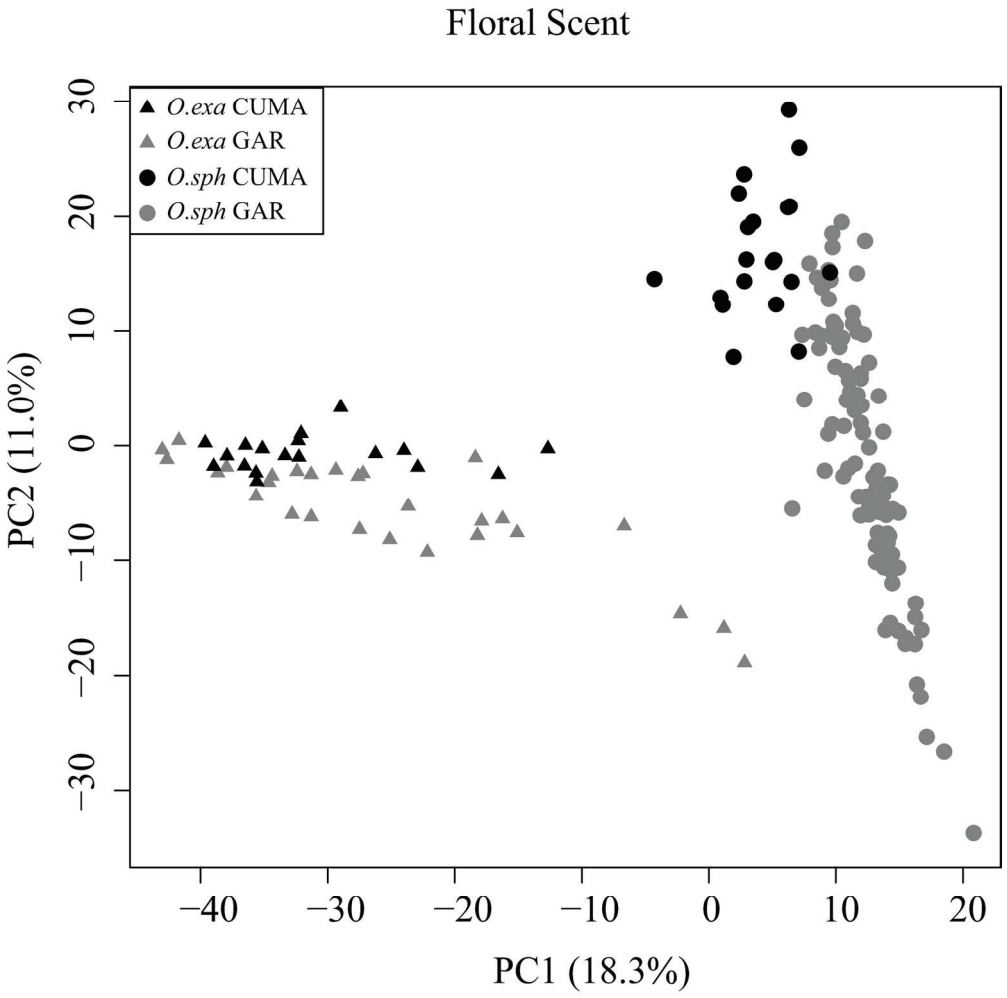
166x173mm (300 x 300 DPI)



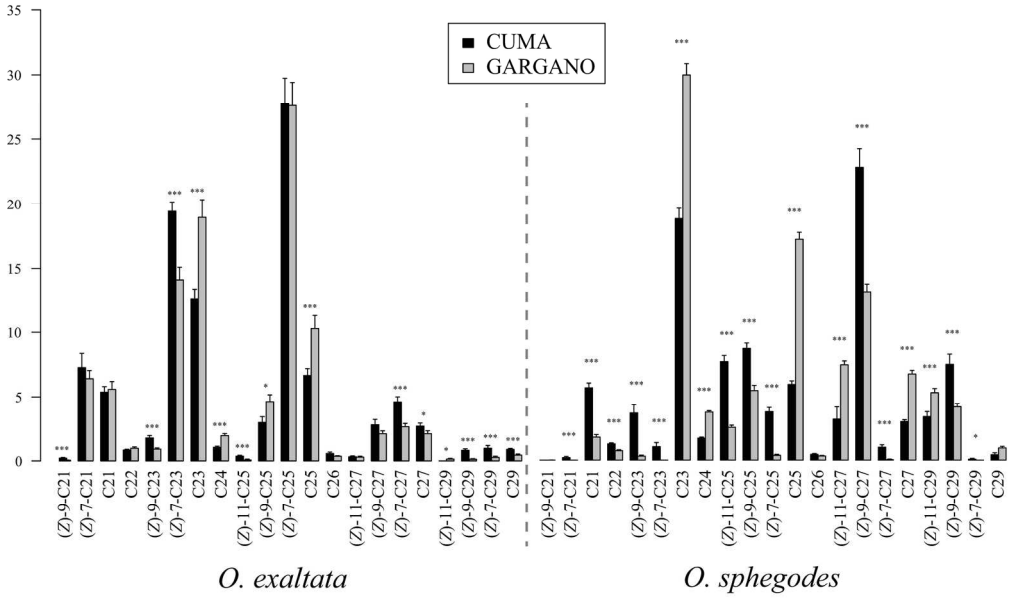
121x112mm (300 x 300 DPI)



110x79mm (300 x 300 DPI)



166x164mm (300 x 300 DPI)



174x104mm (300 x 300 DPI)